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Autoxidation products of both carbohydrates and lipids are increased in uremic plasma: Is there oxidative stress in uremia?

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Autoxidation products of both carbohydrates and lipids are increased in uremic plasma: Is there oxidative stress in uremia?

Background. Advanced glycation end products (AGEs), formed by non-enzymatic glycation and oxidation (glycoxidation) reactions, have been implicated in the pathogenesis of several diseases, including normoglycemic uremia. AGE research in uremia has focused on the accumulation of carbohydrate-derived adducts generated by the Maillard reaction. Recent studies, however, have demonstrated that one AGE, the glycoxidation product carboxymethyllysine (CML), could be derived not only from carbohydrates but also from oxidation of polyunsaturated fatty acids *in vitro*, raising the possibility that both carbohydrate and lipid autoxidation might be increased in uremia.

Methods. To address this hypothesis, we applied gas chromatography-mass spectrometry and high performance liquid chromatography to measure protein adducts formed in uremic plasma by reactions between carbonyl compounds and protein amino groups: pentosidine derived from carbohydrate-derived carbonyls, malondialdehyde (MDA)-lysine derived from lipid-derived carbonyls, and CML originating possibly from both sources.

Results. All three adducts were elevated in uremic plasma. Plasma CML levels were mainly (>95%) albumin bound. Their levels were not correlated with fructoselysine levels and were similar in diabetic and non-diabetic patients on hemodialysis, indicating that their increase was not driven by glucose. Pentosidine and MDA-lysine were also increased in plasma to the same extent in diabetic and non-diabetic hemodialysis patients. Statistical analysis indicated that plasma levels of CML correlated weakly ($P < 0.05$) with those of pentosidine and MDA-lysine, but that pentosidine and MDA-lysine varied independently ($P > 0.5$).

Conclusions. These data suggest that the increased levels of AGEs in blood, and probably in tissues, reported in uremia implicate a broad derangement in non-enzymatic biochemistry involving alterations in autoxidation of both carbohydrates and lipids.

Key words: carboxymethyllysine, pentosidine, malondialdehyde-lysine, glycoxidation, lipoxidation, oxidative protein damage, non-enzymatic biochemistry, AGEs.

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Proteins in the body are continuously modified by non-enzymatic glycation and oxidation (glycoxidation) reactions, also known as Maillard or browning reactions. Schiff base and Amadori adducts between protein amino groups and carbohydrate-derived carbonyls eventually yield advanced glycation end products (AGEs) [1]. AGE modified proteins increase during normal aging, but markedly increase in diabetes and uremia, damaging tissues through alterations of their structure and function [1], and stimulation of pathologic cellular responses [2–6].

The availability of sensitive and specific methods to identify some of the AGEs generated on proteins during the Maillard reaction has helped unravel the various pathways leading to the formation of AGEs in uremia. *In vitro* studies have demonstrated that pentosidine [7] originates from glucose-, ribose-, or ascorbic acid-derived carbonyl intermediates [8–11]. Similarly, N^{ϵ}-(carboxymethyl)lysine (CML) [12] is formed on proteins exposed to glucose or ascorbic acid under oxidative condition [10–13]. Both compounds have been taken as markers of carbohydrate-derived AGE adducts. More recently, however, similar *in vitro* studies have demonstrated that CML can also be derived from lipid sources such as polyunsaturated fatty acids [14], raising the possibility that AGE adducts arise from carbonyl compounds derived from both carbohydrates and lipids.

To test this hypothesis, we have measured in uremic plasma several stable protein adducts formed by reactions between carbonyl compounds and lysine amino groups in proteins [15]: pentosidine originating from carbohydrate-derived carbonyls, malondialdehyde (MDA)-lysine from lipid-derived carbonyls [16], and CML originating possibly from both carbohydrates and lipids. Our results show that there are significant increases in all three compounds, suggesting that the increased levels of AGEs in uremic patients, whether detected by immunohistochemical [11] or enzyme-linked immunosorbent assay (ELISA) techniques

Table 1. Profile of subjects examined in this study

	Group I Normal subjects ($N = 9$)	Group II Non-diabetic HD patients ($N = 25$)	Group III Diabetic HD patients ($N = 21$)
Age *years*	43.5 ± 8.1	60.6 ± 9.9[a]	62.0 ± 9.1[a]
Total protein *g/dl*	7.90 ± 0.32	7.28 ± 0.46[b]	7.16 ± 0.48[a]
Serum creatinine *mg/dl*	0.81 ± 0.11	10.73 ± 2.19[c]	10.10 ± 2.25[a]
Fructoselysine *mmol/mol Lys*	2.27 ± 0.17	2.50 ± 0.58	4.33 ± 1.14[c]
Triglycerides *mg/dl*	88.8 ± 70.5	122.0 ± 91.2	121.7 ± 80.0

HD is hemodialysis.
[a] $P < 0.001$ vs normal subjects
[b] $P < 0.01$ vs normal subjects
[c] $P < 0.001$ vs. normal and non-diabetic HD subjects

[17], result from a broad derangement in non-enzymatic biochemistry in uremia, involving alterations in autoxidation of both carbohydrates and lipids.

METHODS

Plasma samples

Plasma was obtained from 9 normal subjects with normal renal function and no proteinuria (Group I in Table 1), and 25 non-diabetic (Group II) and 21 diabetic patients undergoing hemodialysis (Group III). Characteristics of the study population are summarized in Table 1. None of the patients were on lipid lowering drugs. Informed consent was obtained from all patients. Normal renal function was defined as a serum creatinine level below 1.0 mg/dl. Proteinuria was considered to be absent if no protein was detectable in the urine with Albustix (Bayer-Sankyo, Tokyo, Japan). Hemodialysis was performed three times weekly for four hours with a dialysate containing 2 g/liter glucose, 30 mmol/liter bicarbonate, and 8 mmol/liter acetate. Plasma protein concentration was determined in fresh heparinized plasma using the Bio-Rad protein assay reagent (Richmond, CA, USA) with human albumin as a standard. Fractionation of plasma proteins was performed by gel filtration on a Sephacryl S-200 column (5.0 × 67 cm; Pharmacia, Uppsala, Sweden). Plasma triglycerides and creatinine were measured by routine methods at the central laboratory of Tokai University Hospital.

Materials

Unless otherwise indicated, reagents were of highest quality obtainable from Sigma (St. Louis, MO, USA). The preparation of fructoselysine, CML, pentosidine, and MDA-lysine standards have been described previously [14, 18, 19].

Analytical procedure

For measurement of CML and MDA-lysine, plasma samples (75 μl) were diluted with 75 μl 0.2 M sodium borate, pH 9.2, followed by addition of 15 μl 5.5 M $NaBH_4$ in 0.1 N NaOH (final 500 mM BH_4). Reduction was carried out at 4°C overnight and then protein precipitated by addition of an equal volume of 20% trichloroacetic acid. Protein was pelleted by centrifugation at 2000 × g for three to five minutes. The supernatant was discarded and the pellet washed with 300 μl 10% trichloroacetic acid. Heavy labeled internal standards were added and the sample hydrolyzed in 3 ml 6 N HCl at 110°C for 24 hours. Fructoselysine was analyzed in a separate 75 μl aliquot of plasma prepared as above except that the reduction step was omitted. The hydrolysates were dried by centrifugal evaporation (Savant Speed Vac, Farmingdale, NY, USA). Fructoselysine, CML, MDA-lysine, and lysine in plasma hydrolysates were measured as their N,O-trifluoroacetyl methyl esters by selected-ion monitoring gas chromatography/mass spectrometry (GC/MS), as described previously [14, 18, 19]. Analytes were normalized to the lysine content of the sample, following external standardization using calibration curves generated from solutions containing constant amounts of heavy labeled standards and increasing amounts of unlabeled standard.

Pentosidine was measured in hydrolysates of separate plasma samples after reduction, precipitation and acid hydrolysis as above, except that the final concentration of borohydride used for reduction was decreased to 100 mM. Hydrolysates were dried, dissolved in 10 ml water and applied to a 1 ml SP-Sephadex C-25 (Pharmacia Biotech) column equilibrated in water. The sample eluate was discarded. The column was next eluted with 20 ml 0.1 N HCl, and this eluate was also discarded. Pentosidine was eluted with 5 ml 0.1 N HCl. The eluate was dried and an aliquot then used for quantification of pentosidine by reversed-phase high performance liquid chromatography (HPLC) using post-column fluorescence detection (Ex = 328 nm, Em = 378 nm), as described previously [19]. A second aliquot of the SP-Sephadex eluate was used to measure lysine content by amino acid analysis, and pentosidine concentration was expressed relative to lysine content.

All samples assayed in this study were analyzed in single batches to exclude interassay variation. Intraassay coefficients of variation of the assays were determined by repeat analyses ($N = 5$) of low (normal) and high (uremic) sample pools. Coefficients of variation ranged from 5 to 15% for the various assays, where the lower number refers to the

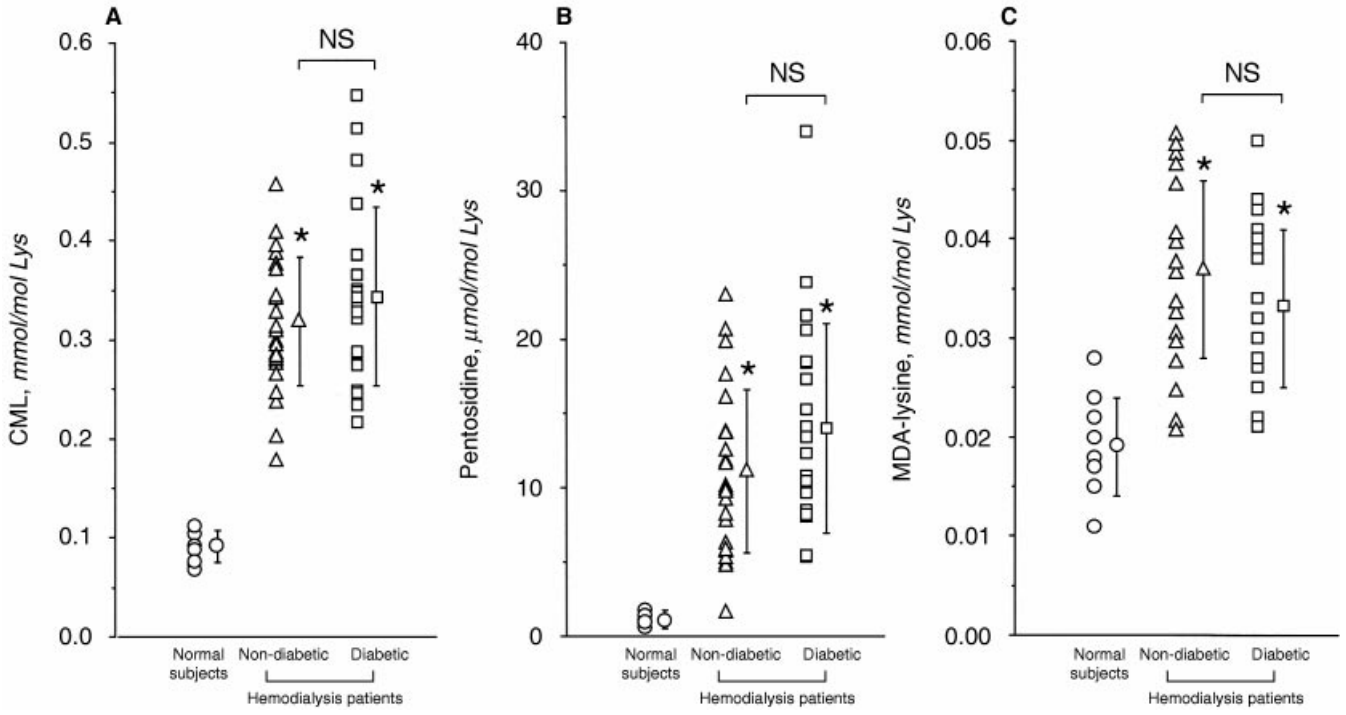

Fig. 1. Plasma N^{ϵ}-(carboxymethyl)lysine (CML), pentosidine, and MDA-lysine levels in normal and hemodialysis subjects. CML (*A*), pentosidine (*B*), and malondialdehyde (MDA)-lysine (*C*) levels in acid hydrolysates of plasma from normal subjects (Group I, $N = 9$), non-diabetic hemodialysis patients (Group II, $N = 25$), and diabetic hemodialysis patients (Group III, $N = 21$), were determined by GC/MS or HPLC assay (detailed in **Methods** section). Data are expressed as means ± SD. *$P < 0.0001$ versus normal subjects. NS is not significant.

coefficients of variation for the high standard and the higher number refers to the coefficients of variation for the low standard: pentosidine, 5 to 13%; CML, 5 to 9%; and MDA-lysine, 9 to 15%.

Statistical analysis

Data are expressed as means ± SD. The Student's *t*-test was used for a statistical evaluation of significant difference between the two groups. Correlation was assessed by linear regression analysis.

RESULTS

As shown in Table 1, hemodialysis patients had slightly lower protein and significantly higher serum creatinine concentrations. Neither of these measures differed between the non-diabetic and diabetic hemodialysis patients. Fructoselysine was increased approximately two-fold in diabetic hemodialysis patients but was unchanged in non-diabetic hemodialysis patients, compared to controls. Triglycerides were similar in all three groups.

Results of measurement of CML, pentosidine and MDA-lysine in plasma are shown in Figure 1. Plasma CML levels (Fig. 1A) of non-diabetic (Group II in Table 1) and diabetic hemodialysis patients (Group III) were significantly higher than those of normal subjects (Group I) (0.313 ± 0.065 and 0.342 ± 0.090 vs. 0.090 ± 0.016 mmol/mol Lys, respectively, $P < 0.0001$). They were not significantly different between the non-diabetic and diabetic hemodialysis patients, a finding interpreted as evidence that CML accumulation is not driven by increased glucose concentrations. This conclusion is also supported by the lack of correlation between CML and fructoselysine (Fig. 2A). Thus, fructoselysine is a known precursor of CML *in vitro*, and its concentration was increased about twofold in the diabetic hemodialysis patients (Table 1).

Plasma pentosidine levels (Fig. 1B) of non-diabetic and diabetic hemodialysis patients were also significantly higher than those of normal subjects (11.12 ± 5.46 and 14.02 ± 7.04 vs. 1.13 ± 0.36 μmol/mol Lys, respectively, $P < 0.0001$). They were not significantly different between non-diabetic and diabetic hemodialysis patients. Levels of pentosidine did not correlate with blood glucose or fructoselysine.

CML and pentosidine, both glycoxidation products, were significantly correlated with each other in all hemodialysis patients (Fig. 2B). In order to determine which plasma protein fraction(s) was post-translationally modified with CML, plasma was fractionated by gel filtration and the CML content was determined in the albumin and IgG fractions of a pooled plasma sample. CML values for total plasma, albumin, and IgG were 0.306, 0.576, 0.189 mmol/mol Lys, respectively. Since albumin constitutes the major

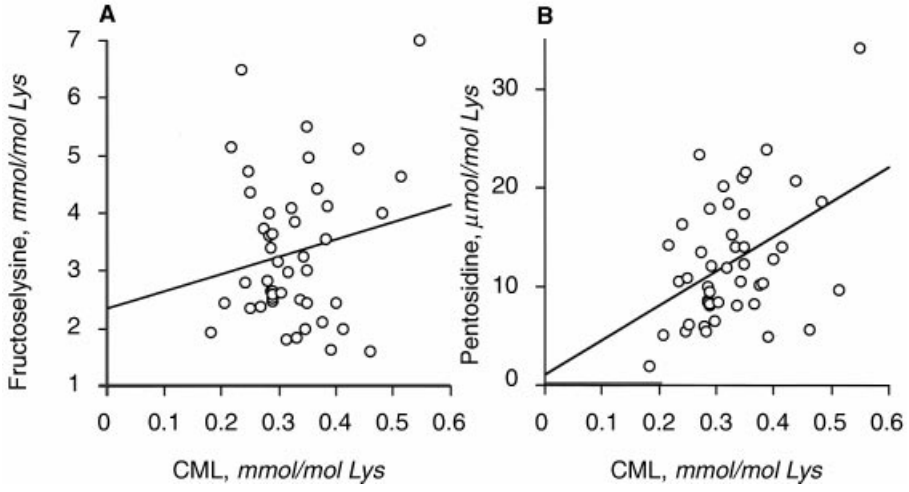

Fig. 2. (*A*) Correlation between plasma CML and fructoselysine in hemodialysis patients. Plasma CML levels were not correlated with those of fructoselysine in hemodialysis subjects ($N = 46$, $r = 0.186$, $P = 0.216$). The equation of the line is y = 3.022x + 2.345. (*B*) Correlation between plasma CML and pentosidine in hemodialysis patients. Plasma CML levels were correlated with those of pentosidine in hemodialysis subjects ($N = 46$, $r = 0.430$, $P < 0.01$). The equation of the line is y = 34.86x + 1.07.

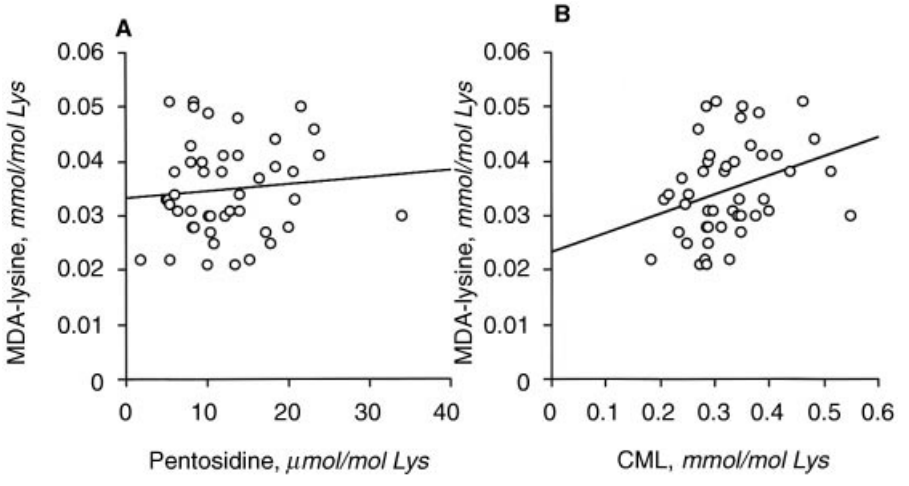

Fig. 3. (*A*) Correlation between plasma pentosidine and MDA-lysine in hemodialysis patients. Plasma pentosidine levels were not correlated with those of MDA-lysine in hemodialysis subjects ($N = 46$, $r = 0.097$, $P = 0.522$). The equation of the line is y = 0.00013x + 0.033. (*B*) Correlation between plasma CML and MDA-lysine in hemodialysis patients. Plasma CML levels were correlated with those of MDA-lysine in hemodialysis subjects ($N = 46$, $r = 0.320$, $P < 0.05$). The equation of the line is y = 0.035x + 0.023.

protein in plasma (~40 mg albumin out of ~70 mg total plasma protein/ml), these data indicate that over 95% of plasma CML is associated with the albumin fraction, a finding similar to that previously reported for pentosidine [20].

Levels of MDA-lysine levels (Fig. 1C) in plasma proteins of non-diabetic and diabetic hemodialysis patients were also significantly higher than those of normal subjects (0.037 ± 0.009 and 0.033 ± 0.008 vs. 0.019 ± 0.005 mmol/mol Lys, respectively, $P < 0.0001$). Like CML and pentosidine, they were not significantly different between diabetic and non-diabetic hemodialysis patients. Levels of MDA-lysine were not correlated with those of triglycerides in hemodialysis patients ($r = -0.329$, $P = 0.467$).

Plasma levels of CML, but not pentosidine (Fig. 3A), were correlated with MDA levels in hemodialysis patients (Fig. 3B), although levels of CML and pentosidine were correlated weakly with one another.

DISCUSSION

Research on AGEs in uremia has focused on the accumulation of carbohydrate-derived adducts generated by the Maillard reaction, such as, AGE-peptides [17, 21], pentosidine [20, 22, 23], and CML [24]. We have recently presented evidence that AGE generation in uremic plasma is stimulated by the accumulation of carbonyl intermediates resulting from the oxidation of glucose and ascorbic acid [11, 25]. We now demonstrate the parallel accumulation of another carbonyl compound, MDA, derived from lipid peroxidation. The accumulation in uremic circulation of carbonyl intermediates derived from various sources, such as carbohydrates, ascorbic acids, and lipids, may be described as "carbonyl stress."

The finding of elevated levels of MDA-lysine in uremic sera is of interest. Only indirect evidence on changes in MDA are available, based on measurement of thiobarbituric acid reactive substances (TBARS) whose levels were reported to be either elevated [26] or normal [27] in uremic sera. TBARS measurements are not specific for MDA as they include several other compounds, depending on assay conditions, including prostaglandin and leukotriene metabolites [28]. The present GC/MS methodology is highly sensitive and specific for MDA-lysine adducts in proteins. Unlike TBARS, it evaluates actual protein modification

resulting from lipid peroxidation. Although the polyunsaturated fatty acid composition of plasma was not measured in this study, the lack of a relationship between triglyceride levels and MDA-lysine or CML, among all subjects, suggests that increased polyunsaturated fatty acid oxidation was a major contributor to MDA-lysine formation.

The weak correlations of CML with MDA-lysine, and CML with pentosidine suggests that CML is derived not only from carbohydrate-autoxidation products of the Maillard reaction, but also from lipid peroxidation products. Fu et al recently demonstrated the generation of CML from polyunsaturated fatty acids [14]. The lack of correlation between MDA-lysine and pentosidine is consistent with the observation that pentosidine originates solely from carbohydrate [8, 9] and MDA-lysine from lipid peroxidation. In addition to MDA, a wide variety of other lipid-derived carbonyl compounds might react with proteins and deserve further study [29].

AGEs such as pentosidine and CML, as well as MDA-lysine, are formed by carbonyl-amine chemistry between protein amino- and guanidino-groups and carbohydrate- or lipid-derived carbonyl compounds. The identity of the carbonyl compounds accumulating in uremia remains a matter of speculation. Several substances are probably involved as no single carbonyl precursor is known to result in the simultaneous formation of CML, pentosidine, and MDA-lysine. The lack of difference between diabetic and non-diabetic uremic plasma suggests that it is not glucose. It cannot be ascorbate/dehydroascorbate whose level is decreased in uremic plasma [11]. Another intermediate compound formed during Maillard reaction, 3-deoxyglucosone, is known to accumulate in uremia [30], but is unlikely to be a candidate. Addition of 3-deoxyglucosone to normal plasma, even at concentrations higher than those reported in uremic subjects, failed to raise pentosidine concentrations during *in vitro* incubation [25].

The present results demonstrate that, like pentosidine, over 90% of CML is bound to albumin. The two best characterized AGE adducts are thus associated with high molecular weight substances in the serum, a finding in contrast to that reported for immunologically detected AGE-peptides (molecular weight, < 10 kD) [17]. Although most, if not all polyclonal anti-AGE antibodies recognize CML [31, 32], the one used in studies of AGE-peptides apparently recognizes another as yet unknown epitope. Until the identity of the epitope, as well as the origin of these small fragments are elucidated, it will be difficult to integrate immunological observations into a broader view of carbonyl stress in uremia.

The reasons for the elevation of carbonyl compounds in uremia remain to be elucidated. It might be due to the retention of various metabolites as a result of kidney failure, that is, either defective catabolism through glomerular filtration and subsequent tubular reabsorption and destruction as demonstrated for pentosidine [33] or impaired removal through urinary excretion.

Alternatively, a uremia-associated oxidative stress [34–39] might augment the production of carbonyl compounds as already suggested for CML generation from glyoxal or dehydroascorbate, and for pentosidine generation from arabinose or dehydroascorbate [10, 11]. The respective contributions of these two pathways require further clarification.

Whatever the identity of the carbonyl compounds and the mechanisms of their rise in uremia, we suggest that identifying the various AGE adducts as well as MDA-lysine as "carbonyl stress end products" underlies that the non-enzymatic modifications disorders observed in uremia extend beyond the borders of carbohydrate-derived Maillard reaction adducts.

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APPENDIX

Abbreviations used in this paper: AGEs, advanced glycation end products; CML, N^{ϵ}-(carboxymethyl)lysine; ELISA, enzyme-linked immonosorbent assay; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances.

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